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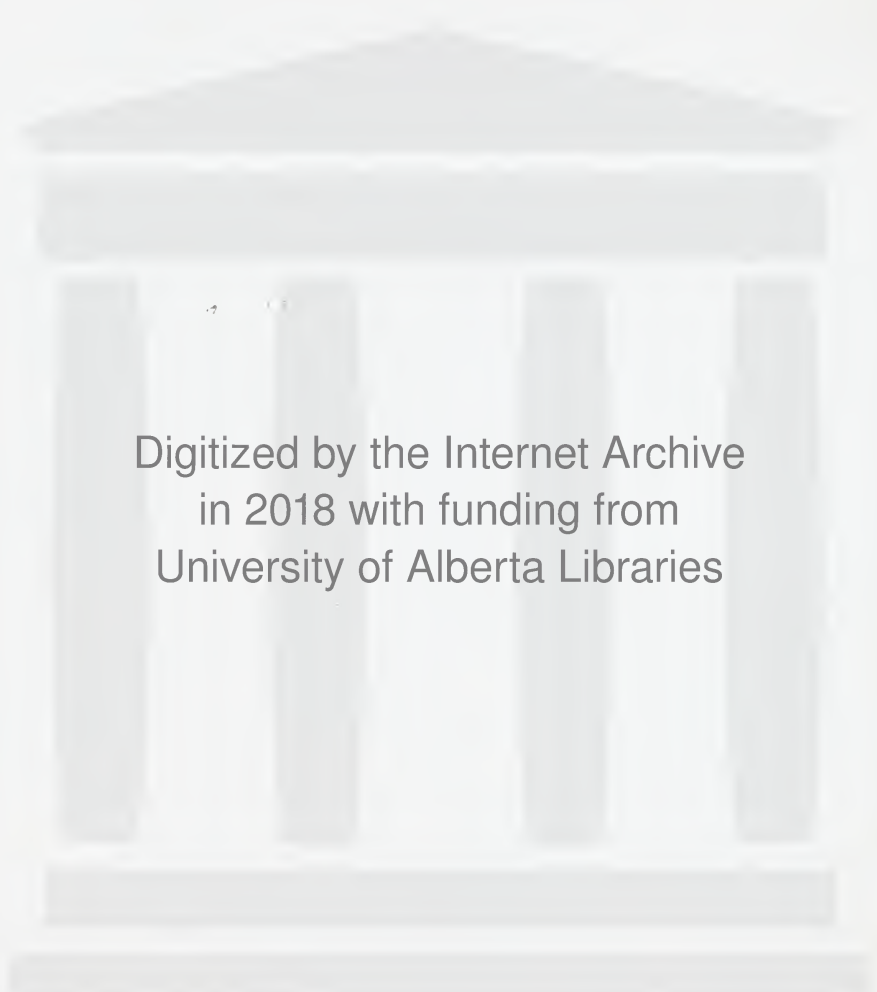
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GLYOXALINES IN URINE.

Terrence M. Raragosky.

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GLYOXALINES IN URINE

Terrence Maurice Raragosky

A thesis submitted in conformity with the
requirements for the degree of Master of Science
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Department of Biochemistry

University of Alberta

1941

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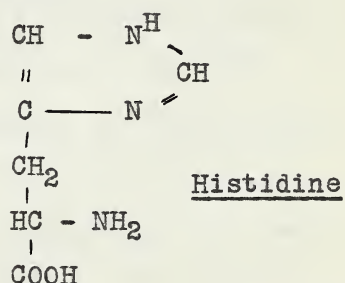
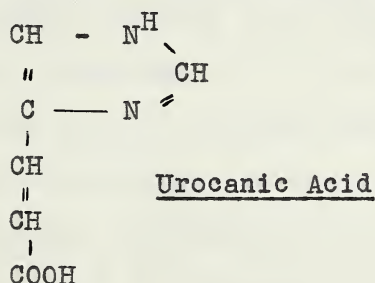
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GLYOXALINES IN URINE

I Introduction and Scope

Ever since it was found that nearly all urines gave an orange or red color in an alkaline solution of diazotized sulphanilic acid, there have been periodic attempts to isolate the substance or substances responsible for the reaction. Ehrlich⁽¹⁾ who first used the test, was interested in an intense color given by the urine of typhoid patients, but this substance has not yet been isolated. Several color-giving substances, however, have been isolated from urine and definitely identified. The first was urocanic acid, isolated from dog's urine and identified, first by Jaffe⁽²⁾ in 1874, and later by Siegfried⁽³⁾ (1898).



The second diazo chromogen to be isolated and identified was Histidine, obtained in small amount from large volumes of human urine by Engeland in 1908⁽⁴⁾. Hunter, in 1922⁽⁵⁾, was also able to isolate 0.1 gram of histidine monohydrochloride from a litre of measles

urine in which, it was estimated 0.31 gram of the material were present. Armstrong and Walker⁽⁶⁾ were also able to isolate a small amount of Histidine from 10 litres of pregnancy urine. Ackermann and Fuchs⁽⁷⁾ in 1939, obtained from 10 litres of mixed urine, 4.6 grams of Histidine dipicrolonate and 11.7 mgm.

Histamine .

The above are the iminazoles whose presence in urine has been definitely established and it is significant to note that in the majority of the cases the urines from which the glyoxalines were isolated were of a pathological nature.

There have been, further, several suggestions as to the presence of other as yet unidentified iminazoles in urines. Fürth and Majer⁽⁸⁾ mention that Kutscher isolated from human urine iminazole acetic acid. Engeland, in his noteworthy paper of 1908, stated that he isolated small amounts of crystalline diazo-chromogens, which, on the basis of a partial analysis, he believed to be the higher and the lower homologues of Histidine, respectively. He further isolated a substance as the gold salt ($C_{15}H_{36}N_8O_{13} \cdot HCl. AuCl_3$) which gave a strong positive diazo reaction. Hunter⁽⁹⁾, in his work on Carnosin in cat muscle and iminazole excretion in urine, has remarked on the presence, in a purified fraction of cat's urine, of a diazo-chromogen in some respects similar to urocanic acid but whose nature is as yet unknown.

From the foregoing discussion it can be seen that there is a variety of iminazoles that may be present in both normal and pathological urines.

Paralleling the interest in the isolation of glyoxaline compounds, there have been numerous studies on methods of estimation of those compounds in urine. Diazo-benzene-p-sulphonic acid dissolved in sodium carbonate, as recommended by Pauly (1904)⁽¹⁰⁾, has been the most widely used reagent in biochemical work for the detection of iminazoles.

In 1914, Weiss and Ssobolew⁽¹¹⁾ published a method for the estimation of Histidine which was based upon the well-known reaction of Pauly. This method was subsequently applied by Masslow⁽¹²⁾ in a series of metabolism experiments on dogs and by Fürth⁽¹³⁾ to a somewhat similar series in man. These were possibly the earliest attempts to estimate iminazole compounds in urines.

Koessler and Hanke in 1919⁽¹⁴⁾, by the use of their modification of the diazo reagent, devised a method which they showed to be accurate for the estimation of histidine, histamine and other iminazoles. In a later paper⁽¹⁵⁾ (1924) they applied this method to the colorimetric determination of iminazole compounds in urine. The urine to be examined was first treated by the lead fractionation technique of Weiss and the diazo test was applied to the 3rd or iminazole fraction of urine. Subsequently, this method was applied, in

whole or in slightly modified form, by several authors.

Hunter(9)(16) observed that the above method was suitable for the routine estimation of iminazoles in urine and commented on the indispensability of the preliminary fractionation of the urine and the subsequent evaporation of the filtrate in obtaining maximal color development in the diazo colorimetry. Lelu (1936)(17) applied the Koessler-Hanke method in her study on metabolism of iminazoles. In a critical analysis of the Koessler-Hanke method, Lelu came to the conclusion that the method was not an absolute measure of the iminazole content but merely a good approximation. Other authors, of whom a good bibliography is given by Tschopp and Tschopp(18) have used the Koessler-Hanke method either in its original form or after slight modification.

Scope of the Work

The present study has resolved itself into two main topics: firstly, the development of a satisfactory method for the estimation of urinary iminazole, and secondly, the study of the nature of the iminazoles present in human urine. With these we shall now proceed to deal.

II The Estimation of Urinary Iminazole

Preliminary Fractionation of the Urine

The prime consideration was to develop a simple, rapid procedure that could be readily applied to the routine analysis of urine for clinical laboratory purposes.

Secondly, we attempted to standardize the method of fractionation so that it might be applicable to all cases without the necessity for determining for each individual urine the optimal quantities of reagents to be used.

It has been pointed out by several authors (Weiss, Hunter, Koessler and Hanke, etc.) that, in order to estimate the urinary iminazole with any degree of accuracy the diazo test must be applied to a suitably purified fraction of urine. The urine must be treated to remove, as completely as possible, not only those substances whose presence has an inhibitory effect on color development, but also those substances, other than iminazoles, that give a positive reaction with the diazo reagent. Such a fraction, suitable for the application of the diazo reagents may be prepared from urine by the lead fractionation technique first developed by Weiss⁽¹⁹⁾ and later used by Koessler and Hanke⁽¹⁵⁾ in their study of urinary iminazoles. The method used by these latter authors forms the basis of our own technique which is outlined below.

Experimental

(a) Collection of the Urine - The urine samples were collected in 24-hour volumes, the results there being expressed in mgm. iminazole excreted in 24 hours. Where such procedure was possible the 24 hour-sample was preferably collected in 2-12-hour portions: a day portion (8 a.m. - 8 p.m.), and a night portion (8 p.m. -

8 a.m.). Determinations were carried out on both portions, the results on these 12-hour portions giving us some insight into the nature of the metabolism and excretion of the iminazole compounds.

The urines, so collected, were placed in well stoppered bottles, covered with a layer of toluene and placed in the refrigerator. By such means of preservation, it was found that the urinary iminazole content remained unchanged for long periods of time.

Reagents used - The reagents and the method used were essentially similar to those employed by Koessler and Hanke (1924) but certain modifications have been made to make this method more applicable as a standard procedure. Instead of using a saturated solution of normal lead acetate, we used a 40% solution since the saturated solution is of necessity a variable concentration and hence unsuited to a standard procedure of the type under consideration. The NaOH used was a 2.0 N. solution, the normality being accurate to the first decimal place.

It might be mentioned at this point that we attempted the use of Goulard's extract (U.S.P.)⁽²⁰⁾ in the fractionation procedure. This was later abandoned when it was found, on experiment, that the results were not as good as those obtainable by the use of normal lead acetate and sodium hydroxide. It was observed that the iminazole fractions remained

somewhat colored despite the use of relatively large amounts of the extract. Also in the subsequent diazo-colorimetry the colors obtained were yellower and of weaker intensity than in the case of iminazole fractions prepared by the use of normal lead acetate and NaOH. We therefore stayed with the latter procedure.

Method Employed - From the 12- or 24-hour specimen of urine, exactly 5 c.c. were pipetted into a tapered 15 ml. centrifuge tube. To this was added 4.0 ml. 40% normal lead acetate solution and the contents well mixed. We then added 4.0 ml. 2 N. NaOH solution and the contents again thoroughly mixed. The tube was then placed in the centrifuge for 2-5 minutes to bring about clear separation. The voluminous lead precipitate carried down with it urine pigments, organic and inorganic acids, purines, other urinary chromogens and certain substances that inhibit coupling with the diazo reagents, but it removed none of the glyoxalines (Koessler and Hanke⁽¹⁵⁾, Lelu⁽¹⁷⁾).

The supernatant solution was decanted off into a larger centrifuge tube (50 ml.) and the lead precipitate was washed twice, using each time 2-3 c.c. of distilled water. The washings were added to the filtrate. To this latter was added sufficient disodium hydrogen phosphate solution to completely remove all excess lead. It was found that an excess of Na_2HPO_4 may be added, the salt not interfering with the diazo test, while it is imperative to remove all traces of

lead, its presence in solution causes clouding with Na_2CO_3 subsequently used in the colorimetry. The resultant lead phosphate precipitate was centrifuged off, the filtrate decanted into a small erlenmeyer flask (125 ml.) and the precipitate was washed with distilled water to which a few drops of Na_2HPO_4 solution were added. By this procedure, lead fractions were obtained that were in almost every case quite clear and colorless. These latter were the criteria of successful fractionation.

The filtrate and washings were now evaporated nearly to dryness, made up to 10 ml. and aliquot portions of this solution were used for the estimation of iminazole. The mode of evaporation was found to have a marked influence over both the intensity and the quality of the color produced. In all, 4 methods of heat treatment were investigated:

- (1) Evaporation at reduced pressure (Temperature $40^\circ\text{C}.$)
- (2) Evaporation on a boiling water bath (Temperature $92^\circ\text{C}.$)
- (3) Boiling the filtrate to near-dryness on the hot plate (Temperature approximately $110^\circ\text{C}.$)
- (4) Refluxing the filtrate for various periods of time ($\frac{1}{2}$ -2 hours) and subsequently evaporation to low volume on the hot plate.

It was found that the samples treated by the first two methods yielded definitely poorer results than those treated by the latter two methods. The method

finally adapted consisted in boiling the lead filtrate to near-dryness on the hot plate (till crystals began to appear in the boiling solution). The flask was then removed from the plate, about 10 c.c. distilled water added and this solution once more evaporated as before to near-dryness. The residue was transferred to a 10 c.c. volumetric flask and made up to the mark with careful washings of the erlenmeyer flask. This resultant solution was then subjected to diazo colorimetry for the estimation of iminazoles.

In the present method, three conditions were taken as criteria of the efficiency of fractionation of the urine, quality of color development, intensity of color developed and proportionality of the color readings. The latter seemed to be of the greatest value in assessing the fractionation method. Two factors were found to influence these criteria.

The first factor was the amount of lead acetate used in the fractionation. In a sample of normal urine increasing amounts of lead acetate were used. The results are given below (5 ml. samples of normal urine were used in each case):

Urine 1:	PbOAc - 2.5 ml.	→ 7.8 mm.	(yellow)
	3.0 "	→ 9.6 "	(pink)
Urine 2:	PbOAc - 3.0 "	→ 7.4 "	(yellowish)
	4.0 "	→ 8.2 "	(pink)

It can be seen that there was an increase in intensity of color developed, and an improvement both in

the quality of the color obtained and the proportionality of the readings. With the use of larger amounts of lead acetate, the colors obtained in the subsequent diazo test tended to be less yellow and more pink.

The second factor affecting these criteria was the degree of heat applied in the evaporation of the crude filtrate. Higher degrees of heat and concentration were found to bring about the more complete removal of the volatile constituents of the urine. Histidine in presence of ammonia and in contact with the diazo reagent will give a yellow color. Phenol, Indol, Sc^atol and other related volatile phenolic substances that can by their presence alter the quality or inhibit color development were more or less completely removed by the heat treatment. The degree of success was guaged by the results obtained, i.e. in regard to the criteria mentioned.

Method of Heat Treatment	Approx. Temp.	Quality	Readings
Evaporation at reduced pressure	50°C.	Yellow-orange	7.4
Evaporation on the boiling water bath	94°C.	Better but Yellow	9.2
Evaporation by boiling to dryness, timed on hot plate	105-110°C.	Pure Pink	#10.3
Evaporation by refluxing (1½ hours) and evaporation on hot plate	105-110°C.	Pure Pink	#10.4

Intensity of color, quality of color and proportionality of readings are adversely affected by the presence of inhibition substances in the lead fraction. The greater amount of lead acetate used acts to cause more complete precipitation of these substances. The greater degree of heat similarly removes the volatile inhibiting substances. The increase, to optimal values, of our three criteria indicated most satisfactory lead fractionation treatment.

(b) Diazo Colorimetry - Aliquot portions of the lead filtrate were subjected to the Diazo test. The Koessler-Hanke Diazo reagent was used and the procedure carried out in essentially the same manner as described by these authors. The colors developed were measured against an artificial color standard whose preparation has been previously described by Hunter⁽²¹⁾. The color standard was first standardized or calibrated against a solution of pure Histidine monohydrochloride of known strength. The colors developed from the iminazole in the lead filtrates matched very closely the color of the standard solution.

Procedure - The standard solution was placed in the right-hand cup of the colorimeter. Into the left-hand cup were placed:-

(0.5-x) c.c. of pure distilled water,

2.5 c.c. of a freshly prepared 1.1% sol. of Na_2CO_3 ,

1.0 c.c. of fresh diazo reagent.

Using the plunger of the colorimeter as a mixing rod, the contents of the cup were thoroughly mixed. Exactly 1 minute after the addition of the diazo reagent, x ml. (0.05-0.5 ml.) of the lead filtrate was added. The contents were again mixed in the same manner, the cup inserted in position and set at a depth of 10 mm.

Color development in the left hand cup proceeded rapidly, the stages of development being identical with those of Histidine in similar strength of solution. There was first a greenish coloration grading through to yellow, orange, red-orange and finally a clear pink. It was found that maximal color development occurred 3.5-4 minutes after the addition of the test solution. Fading of the color began after a variable interval, depending both on the intensity of the color developed and on the amount of the test solution added. The significant point is that readings are not reliable if taken at intervals other than those specified.

Calculation Results - The urinary glyoxaline was found to resemble very closely, in both properties and behavior, the characteristics of Histidine. Thus it was decided to express urinary glyoxalin in terms of Histidine rather than in terms of a vague iminazole complex as suggested by Koessler and Hanke.

The color standard used was standardized against a known solution of histidine monohydrochloride. It was found that 1 mm. depth read in the colorimeter was

equivalent to 0.00068 mg. Histidine or 0.00092 mg. Histidine monohydrochloride. Thus if we obtained, with a 0.1 ml. aliquot portion, a reading of 11.5 mm. of standard, then: -

0.1 c.c. solution = 11.5 mm.
 1 c.c. solution = 115 mm.
 1 c.c. urine = 115 x 2 mm. = 230 x 0.00068 mg.
 of Histidine per c.c. of that
 particular urine.

The volume of the urine being known, the calculation of the total excretion for the 12 or 24 hour period was a simple matter.

Knoop Tests of Lead Filtrates - Kapeller-Adler⁽²²⁾ has reported the details and modifications of a method for the estimation of Histidine in urine. The test is a modification of the Knoop reaction and its specificity for histidine has been adequately demonstrated. The sensitivity of the reaction has been claimed by the author to be as high as 1:50,000 in pure solution and about 1:10,000 in urine.

The method was applied by us, both to lead filtrates and to pure solutions of histidine monohydrochloride. With solution of pure histidine, it was found that we were unable to duplicate the findings of Kapeller-Adler with respect to the sensitivity of the test. We found the test to be much less sensitive than claimed. The method was also applied to lead filtrates of urines. In all cases, even with urines

with high iminazole content, the Kapeller-Adler test was negative. On the assumption that all or a great part of the glyoxalin contained was histidine, we would have expected positive reactions in all cases as the amounts of lead filtrates added were such as to be within the limits of sensitivity of the test.

The consistency of the negative results indicated several possibilities - that the Kapeller-Adler test is not applicable to lead filtrates. That in the latter some interfering substance or substances are present that prevent the occurrence of the reaction and the appearance of the characteristic color - that the main glyoxalin present in the urines is not histidine but some closely related glyoxaline. Since, subsequently, rather large amounts of histidine were isolated from these same urines tested, it may be conceivable that in the native urine the original glyoxalin is not histidine but some labile glyoxalin readily converted to histidine in the course of the isolation procedure.

Studies on Urines - A great deal of time was spent in this research in the routine application of the new method to the estimation of glyoxalins in human urines. An attempt was made to determine or approximate, if possible, the normal range of iminazole excretion in the normal male adult. In all cases, except in the class studies, the determinations were carried out on 2 12-hour samples (day and night samples respectively) collected as described previously, In

this manner, it was thought that some light might be shed on the metabolism of iminazoles.

Three series of studies were carried out: -

- (1) Normal male subjects - unrestricted diet.
- (2) Class studies (Medical students)
 - a. Urines collected for Mosenthal Kidney function test. Diet more or less restricted.
 - b. Urines collected from the same individuals on an unrestricted diet (for Vitamin C determinations).
- (3) A number of pathological urines. Majority of these suffering from some genital dysfunction.

(1) In the normal male urines studied, the results of which are tabulated below, it was found that there existed in the individual case a relatively wide day-to-day variation in the iminazole excretion. There appeared, however, to be a tendency for the 24-hour excretion to remain at a certain level or within a certain range. It was thus possible to speak of high, medium or low iminazole-excreters and to foretell with some degree of accuracy the amount of glyoxalin that could be found in the urine of any one individual for the 24-hour period. The degree of iminazoluria seemed to be a characteristic inherent in the individual, dependent perhaps on some variation in amino-acid metabolism, the nature of which is at the present time unexplained.

In one subject, in whom 17 24-hour samples were studied there was found to be a range of iminazole excretion from 240-458 milligrams (calculated as Histidine) in the 24-hour period. There were occasional high and low values but the great majority were closely clustered about the average value - 300 milligrams/24 hours. In another case only 3 24-hour samples were studied. All showed a high degree of iminazoluria, the values being 656, 670 and 816 mg./24 hours, respectively.

Below are tabulated the results obtained in individuals in whom more than one sample was studied.

Subject	No. of 24-hr. Samples	Average 24-hr. Excretion	Average "Day" Excretion	Average "Night" Excretion
T.M.R.	17	312.6 mg.	157.8 mg.	154.8 mg.
W.L.D.	2	221.1 "	130.7 "	86.3 "
G.A.L.	3	713.8 "	369.3 "	344.5 "
J.G.R.	2	407.3 "	223.0 "	184.3 "

In the above cases the diet was normal and unrestricted so that the values may be said to represent the normal iminazole excretion of that particular individual.

On comparing the values for the "day" and the "night" samples, it was seen that though there was no wide difference, yet there was in every case a larger iminazole excretion in the day specimen than in the night specimen - in some cases the difference being more marked than in others. This fact indicates that

iminazole excretion is influenced by degree of bodily activity and therefore is not entirely endogenous in nature. The influence of diet in the above individuals was not investigated.

(2) Medical Class Studies

Three series of determinations of urinary iminazole were carried out on two classes of Medical students. In one class, two sets of urines were collected and studied, one set collected for the purposes of the Mosenthal Kidney function test and hence under certain dietary restrictions, and the second set collected under no specific dietary restriction (customary diet of the individual). This latter set of urines was preserved with HCl and H_3PO_4 since they had been collected principally for the estimation of the Vitamin C. Previous to the application of the fractionation procedure, these acids were neutralized with 2 N. NaOH . Results obtained were quite satisfactory. In the second class of students, only one set of urines was collected and studied. These also were collected under the dietary restrictions as demanded by the Mosenthal test. Acknowledgement is here made to Mr. A. Nelson who carried out the investigation on this latter set of urines.

The results of these class studies are tabulated in Table I. Since the first two sets of determinations had been carried out on the same individuals, it was thus possible to compare the results and to appreciate

the influence of restricted and unrestricted diet on the excretion of iminazoles.

Class I - (a) 42 urines collected for Mosenthal test.

The glyoxaline contents of the urines collected varied from 80.0-340 mg./ 24 hours. The average value was 181 mg./24 hours. Expressed as milligrams per 100 ml., the glyoxalin content varied from 4.7-26.3 mg.%, the average being 12.8 mg.%.

(b) 46 urines collected under unrestricted diet. The glyoxalin contents of these urines varied from 113-426 mg./24 hours, the average being 227.3 mg./24 hours. Expressed in milligrams per 100 ml., the content varied from 7.1-53.3 mg.%, the average being 21.8 mg.%.

Table I - Urinary Iminazole in 24-hour Volumes

Subject	Class I(a)			Class I(b)		
	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.
1	1435	132.7	mg.% 9.3	1230	140.5	mg.% 11.4
2	1800	135.1	7.5	870	85.2	9.8
3	1893	187.7	9.8	840	292.5	34.8
4	1485	256.0	17.7	1030	257.8	25.0
5	2021	135.4	6.7	1480	265.7	18.0
6	1208	181.3	15.0	1060	190.3	18.0
7	1538	338.0	22.0	920	310.3	33.7
8	-	-	-	1830	268.8	14.7
9	2057	281.2	13.7	810	268.8	33.2

Subject	Class I(a)			Class I(b)		
	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.
10	-	-	mg. %	500	194.1	38.8
11	1871	200.6	10.7	1150	281.5	24.5
12	868	209.4	24.1	1320	330.3	25.0
13	1714	229.7	13.4	2030	298.2	14.7
14	2795	142.3	4.7	780	174.0	22.3
15	2332	190.6	8.2	900	235.0	26.0
16	1060	210.2	19.8	1040	265.9	25.6
17	2904	148.9	4.7	980	229.2	23.4
18	1430	141.8	9.8	1340	196.8	14.7
19	1036	227.7	22.0	800	426.5	53.3
20	893	203.2	22.8	1655	171.1	10.3
21	-	-	-	1250	251.6	20.1
22	1206	219.8	18.2	880	201.1	22.8
23	2523	162.3	6.4	1020	271.7	26.6
24	1021	90.3	8.8	870	146.7	16.9
25	1195	147.3	12.3	1120	274.2	24.5
26	2049	252.6	12.3	1600	287.2	18.0
27	-	-	-	2150	198.8	9.3
28	1138	79.3	7.0	260	51.3	19.7
29	1287	93.1	7.2	590	134.8	24.5
30	1840	187.4	10.2	1100	281.3	25.6
31	-	-	-	1240	128.2	10.3
32	1588	161.7	10.2	860	276.1	32.1
33	1260	175.6	13.9	1000	277.4	27.7
34	1300	216.0	16.6	-	-	-
35	-	-	-	1030	263.4	25.6

Subject	Class I(a)			Class I(b)		
	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.
			mg. %			mg. %
36	-	-	-	1400	205.6	14.7
37	2344	182.2	7.8	1360	207.2	15.2
38	1035	169.2	16.4	900	264.4	29.4
39	-	-	-	650	134.4	20.7
40	1395	168.2	12.1	1980	161.6	8.2
41	1488	147.5	10.2	1400	251.1	18.0
42	1350	137.5	10.2	1800	127.3	7.1
43	1068	280.5	26.3	-	-	-
44	1637	228.1	13.9	1270	290.2	20.8
45	1655	146.4	8.8	-	-	-
46	1371	165.3	12.1	1540	150.8	9.8
47	1007	78.3	7.8	1040	113.2	10.9
48	1190	159.5	13.4	780	230.1	29.5
49	1332	182.1	13.7	930	253.0	27.2
50	936	183.1	19.6	-	-	-

Comparison of these two sets of results showed that in the great majority of instances there was a marked increase in the glyoxalin excretion when the diet was unrestricted. It was possible that many factors could operate to this effect but the most logical conclusion seemed that the dietary change was the main factor responsible for the heightened iminazole excretion.

Comparison of individual cases also showed the tendency, referred to above, of the iminazole excretion of the individual to be at a certain level, be it high or low. In most cases this is fairly well illustrated.

Class II - 35 urines collected for Mosenthal Test.

Results in this third series were essentially similar to the corresponding set in the first class. Variation in iminazole excretion was found to be from 78-447 mg. in the 24-hour volume. The average excretion was 226 mg./24 hours. Expressed as milligrams per 100 ml. the excretion varied from 4.8-31.9 mg.%, the average value being 16.3 mg.%.

Class II

Subject	24-hour Volume (ml.)	Total 24-hour Iminazole (mg.)	mg. Iminazole per 100 ml.
			mg.%
1	1540	229	14.8
2	985	239	24.3
3	1783	220	12.1
4	1333	198	14.8
5	850	172	20.2
6	750	192	25.6
7	1881	181	9.6
8	1468	198	13.5
9	1171	265	22.6
10	1117	272	24.3
11	2536	236	9.3
12	2205	107	4.9

Subject	24-hour Volume (ml.)	Total 24-hour Iminazole (ma.)	mg. Iminazole per 100 ml.
			mg. %
13	1099	230	20.9
14	2000	313	15.7
15	1916	158	8.3
16	1123	210	18.6
17	1285	224	17.5
18	1675	285	17.0
19	1451	194	13.4
20	1750	222	11.3
21	896	185	20.7
22	1858	202	11.0
23	1629	221	13.6
24	1127	352	31.2
25	1610	176	10.9
26	1187	282	24.6
27	1735	375	21.6
28	1489	201	13.5
29	1799	124	6.9
30	2195	175	8.0
31	1300	196	15.1
32	981	314	31.9
33	1771	447	25.2
34	1406	78	5.6
35	2021	230	11.4

In the course of the Mosenthal test, the urines were tested for the usual clinically significant constituents. These figures are all on hand so that

drawing up of correlations between urinary iminazole and any of the urinary constituents may be attempted though this had not been done to date. From the results shown in a recent paper by Langley⁽²³⁾ such a correlation between iminazole and creatinine contents may be a worthwhile undertaking.

Sex difference - There was observed in the limited number of cases available an appreciable difference in the rates of excretion of iminazole in male and female subjects. Under restricted diet the female subjects excreted an average of 106.7 mg. iminazole in 24 hours as compared to 185 mg./24 hours for the males. Under unrestricted diet female subjects excreted an average of 162.2 mg./24 hours as compared to an average of 230 mg./24 hours in the male subjects. The reason for this sex difference is not known, but the explanation probably lies in the greater bodily activity of men as compared to women and to a large extent also in the differences in body mass. This has not yet been fully studied.

(3) Pathological Urines

In the course of the research several pathological urines were examined incidentally rather than as an organized study of such urines. In several instances the results obtained were interesting from a clinical standpoint. Below are outlined some of the cases that were studied and the findings observed.

Miss B. - Complaint - Metrorrhagia and thyroid

hypofunction. The urinary iminazole before treatment (21/2/40) was 78.3 mg./24 hours (10.6 mg. %). After several days' treatment with thyroid extract, the urinary iminazole rose to 381.9 mg./24 hours (65.3 mg. %). The findings in this case tended to show that the Basal metabolic rate is closely associated with the iminazole excretion. However, since this was an isolated case, no reliable conclusions could be drawn though a clue is given for further study.

Mrs. F. R. - Pregnant - 5 months. Threatened abortion was being treated with Leutocylin injections (Progesterone). In all, 14 samples of urine were analysed for iminazole content. The results of these analyses together with notes on the treatment and the progress of the symptoms are tabulated in Table II.

From the observations made it seemed permissible to draw certain conclusions. With threatened abortion, the iminazole excretion, high in normal pregnancy, dropped markedly and approached the normal female level. On treatment with Leutocylin that tended to stabilize the pregnancy, the iminazole excretion rose again to high levels. Withdrawal of Leutocylin caused a fall in the rate of excretions of iminazole. The findings in this case were in accord with the findings of other authors who have investigated histidinuria in pregnancy. (Kapeller-Adler, Tschopp and Tschopp, Armstrong, and Walker, Fürth and Majer). They have reported that there exists in pregnancy a marked Histidinuria.

Table II - Mrs. F. R. - Habitual abortion
Pregnant - 5 months
Threatened abortion

Date	Total 24-hr. Volume (ml.)	Iminazole Content		Remarks on Condition and Treatment
		Total 24-hr. (mg.)	mg. per 100 ml. (mg.%)	
18/3/40	-	-	-	Vaginal bleeding. 2 ampoules of Leutocylin.
19/3/40	-	-	-	Vaginal bleeding. 1 ampoule of Leutocylin.
20/3/40	-	-	-	Vaginal bleeding. 1 ampoule of Leutocylin.
21/3/40	760	227.4	29.9	10 mg. Leutocylin (Progesterone)
22/3/40	850	346.8	40.8	" "
23/3/40	1435	429.4	29.8	" "
24/3/40	1200	391.7	32.6	" "
25/3/40	950	444.5	46.4	No Leutocylin given
26/3/40	1295	465.0	35.9	10 mg. Leutocylin
27/3/40	520	174.1	33.5	none given
28/3/40	1100	383.0	34.8	" "
30/3/40	1460	532.1	36.5	10 mg. Leutocylin
31/3/40	-	-	-	Bleeding had improved - ceased for the last few days. Patient discharged. Quite well. However, patient readmitted April 23.
23/4/40	-	-	-	Vaginal bleeding. 1 ampoule
24/4/40	280	128.0	45.7	" " no Leutocylin
25/4/40	770	393.8	51.1	" " 1 ampoule "
26/4/40	620	279.8	45.1	No bleeding. No Leutocylin
27/4/40	1080	499.4	46.2	Quiet day. No bleeding. Leutocylin, 1 ampoule.
28/4/40	1500	457.0	30.5	Uterine contractions. Pain in back. Given morphia for pain. No bleeding.
29/4/40	868	160.6	18.4	Severe pains. Yellowish dis- charge per Vaginam. . Labor set in. Leutocylin given. Premature delivery (6 months). Child weigh 2 lbs. 10 oz. at birth - died 2 days later.

Several other urines of a pathological nature were studied. Unfortunately little more was known about these cases than merely the clinical diagnosis, so that the value of the results is necessarily limited. These cases are listed below:

Date	Patient	Diagnosis	Glyoxalin Excretion	
			Total 24-hour Excretion	Excretion in mg. %
21/2/40	Mr. M.	Cushing's syndrome	mg. 111.9	9.2
22/2/40	" "	" "	199.3	17.4
21/2/40	Mr. S.	Male climacteric	82.3	6.25
29/2/40	" "	" "	94.2	6.98

Clinical Significance of Iminazoluria

As can be seen from the above, the extent of the work done on the clinical significance of the test has been very limited. Various authors have reported at length on this aspect of the subject. For a good review of the recent work done on this aspect of the problem, one is referred to the papers by Tschopp and Tschopp⁽¹⁸⁾ or Fürth and Majer⁽⁸⁾.

III Isolation of the Urinary Iminazole

After the development of a satisfactory method of estimation of the iminazoles in urine, we undertook to study the nature of these substances. The assumption that the iminazole was wholly or at least in part, histidine, served as a guide in the isolation procedure.

The process was designed as if the total glyoxalin content were histidine since earlier observation tended to support such a conclusion. The procedure is described below, one of the several isolations done serving as an example.

The urine used was Sample G.A.L. - volume = 3250 ml. By the method of estimation, the glyoxalin content was found to be 1386 mg. (expressed as Histidine base). This urine was placed in a 12 litre round-bottom pyrex flask and to it was added $\frac{1}{2}$ its volume (1625 ml.) of 40% Normal Lead Acetate solution and then an equal volume of 2 N. Sodium Hydroxide. Both these solutions were added slowly with constant stirring to insure thorough mixture of solutions and optimal precipitation. The flask was then stoppered lightly and placed in the refrigerator overnight to allow the precipitate to settle.

In the morning the supernatant, a clear solution of a very faint yellow tinge was carefully siphoned off. The remainder of the mixture was centrifuged in large 250 ml. cups and the supernatant added to the filtrate. The flask was well washed with distilled water and these washings were in turn used to wash the precipitates in the cups. The washing was carried out by thoroughly dispersing the precipitate in the wash water. The mixture was then centrifuged and the supernatant added to the filtrate. Two such washings were done after which the precipitate was discarded.

The combined filtrate and washings were now freed of excess lead ion by the addition of a solution of disodium hydrogen phosphate (5%) till precipitation of the lead was complete. In this instance 230 c.c. of the 5% solution were required to completely remove all lead. The lead phosphate precipitate settled out readily in 2 or 3 hours and then as in the previous precipitation, the separation was brought about by siphoning and centrifuging. The lead phosphate was washed with distilled water to which a small amount of Na_2HPO_4 solution had been added.

The filtrate and washings with a combined volume of about 6-7 litres, was evaporated to a low volume in a large vacuum-distillation apparatus. Periodically the distillation was interrupted and the distillate drained off and tested for ammonia by the use of the Nessler reagent. In the early part of the distillation a strongly positive reaction was obtained but became progressively weaker and then negative. The distillation was then discontinued. The volume now was 2000 ml.

An aliquot portion was taken, suitably diluted and the glyoxalin content was determined by the diazo colorimetric method. The glyoxalin content was found to be 1066 mg.

Mercury Fractionation - Several 5 ml. aliquot portions of the lead filtrate were taken and put through mercury fractionation. Varying amounts of Mercuric

Acetate (25%) were added to each of the 5 ml. aliquot portions. The solution was now made just alkaline to litmus with Na_2CO_3 (10%), and the tubes were centrifuged. The filtrates were discarded. The precipitates in the tubes were washed with distilled water and a drop of 10% Na_2CO_3 . The tubes were once more centrifuged and the filtrates discarded. The precipitates were then suspended in distilled water, made acid by addition of H_2SO_4 and then Hydrogen Sulphide was bubbled through to "free" the precipitated glyoxalin of the mercury. The Mercuric Sulphide was centrifuged off, washed and discarded, while the filtrate and washings were evaporated in vacuo (to remove H_2S) to a volume of about 1 ml. When H_2S was completely removed this concentrated solution was made up to 10 ml. and the glyoxalin content was determined. The fraction was also tested for urea and creatinine. All of the aliquot portions were treated in precisely the same manner. The volume of Mercuric Acetate that had brought about the most favorable fractionation of the 5 ml. aliquot, that is, optimal recovery of the glyoxalin and most complete exclusion of urea and creatinine, was added in proportional amount to the main volume of the evaporated lead filtrate. This solution was treated in precisely the same manner as the 5 ml. aliquot.

In this urine, it was found that 240 ml. 25% Mercuric Acetate was the amount required for optimal fractionation of the glyoxaline from the urea and

creatinine. The HgOAc was added and then the solution made just alkaline to litmus with Na_2CO_3 . The precipitate was centrifuged off, washed, suspended in water, acidified and freed with H_2S . The mercuric sulphide was centrifuged off, washed and the combined filtrate and washings evaporated in vacuo to a volume of 130 ml. An aliquot portion was removed, diluted and the glyoxalin content estimated by the diazo method. The content was found to be 630 mg. Thus we had sustained a loss of over 400 milligrams of glyoxalin in the mercury fractionation. (This happened only in this specific instance. In the other isolations very slight loss occurred in the mercury step.)

By the use of the mercury fractionation, all of the glyoxalin can be precipitated and yet leave the great bulk of the urea and creatinine in the filtrate. This is its main value.

Silver Fractionation - For further purification, the glyoxalin solution was passed through a silver fractionation. 10% Silver Nitrate was added to the filtrate resulting from the mercury fractionation. An immediate brownish precipitate of Ag-purines was formed. The silver nitrate was added to the solution till an excess was present as demonstrable by the development of an immediate brown spot-test with Barium hydroxide solution. The purine precipitate was not filtered off at this stage but left in the solution.

From a burette was added saturated $\text{Ba}(\text{OH})_2$ solution till the solution had reached a pH of 5.0. The solution was now centrifuged and the precipitate discarded. At a pH of 5.0 almost none of the glyoxalin was in the precipitate yet the great part of the purines were precipitated. To the filtrate, saturated $\text{Ba}(\text{OH})_2$ was added to a pH of 8.0. The mixture was centrifuged and the filtrate discarded. In the precipitate was present the bulk of the glyoxalin. The glyoxalin contents of the three fractions were found to be:

1. pH 1 - 5.0 = 37.4 mg.
2. pH 5.0 - 8.0 = 645.4 "
3. pH 8.0 -10.0 = 2.2 "

From the results it appeared that the glyoxalin recovered exceeded the amount originally present. This was explained as the effect of the removal of interfering substances and a fuller development of color with the diazo reagent.

Fractions 1 and 3 were discarded. Fraction 2 was suspended in distilled water and H_2S passed in to free the glyoxalin. The Ag_2S precipitate was centrifuged off, washed 2 times with its own volume of distilled water, saturated with H_2S , and discarded. The resultant filtrate and washings were evaporated in vacuo to a volume of about 150 ml. to remove the H_2S and excess water. An aliquot portion was suitably diluted and the glyoxalin content estimated by diazo-colorimetry.

In this instance the content was 645 mg.

Phosphotungstic Acid Precipitation - The method of Hunter and Eagles⁽²⁴⁾ was followed. The solution was made 0.5 Normal with respect to sulphuric acid by the addition of the requisite amount of 10 Normal acid. Then, to the solution, was added a 20% solution of Phosphotungstic Acid (in 0.5 N. H_2SO_4). The reagent was added, with thorough mixing, to the point where further addition caused no precipitation in a test portion of the supernatant. The flask was then lightly stoppered and allowed to stand overnight in the refrigerator for complete precipitation of the glyoxalin to take place. The glyoxalin phosphotungstic precipitate was centrifuged off in the morning, care being taken to keep the temperature of the solution as low as possible throughout the entire procedure. To this end the centrifuge bottles and cups were kept either in the refrigerator or in ice-water until required. The precipitate was washed two times with its own volume of ice-cold 0.5 N. H_2SO_4 to which had been added some of the Phosphotungstic acid solution (5 ml./100 ml. of 0.5 N. H_2SO_4).

The precipitate of glyoxalin phosphotungstate was transferred to a glass mortar and there thoroughly mixed with solid Barium hydroxide, the latter being added till the supernatant solution tested definitely alkaline to litmus. This mixture was then centrifuged and the filtrates decanted through a filter. Thorough

washing of the precipitate was carried out three times, the precipitate being thoroughly shaken up and dispersed in 0.1 N. $\text{Ba}(\text{OH})_2$ solution. The washings were treated in the same manner as the original filtrate - centrifuged and decanted through filter.

The strongly alkaline filtrate and washings were transferred to an erlenmeyer flask and heated to near boiling. 5 N. H_2SO_4 was added till no further precipitation occurred. This Barium sulphate precipitate was likewise centrifuged off, washed two times with hot distilled water and discarded.

The filtrate and washings were evaporated in vacuo to a volume of about 100 ml. Quantitative removal of both Ba^{++} and SO_4^{--} ions was effected by the addition of the required volumes of 0.1 N. solution of $\text{Ba}(\text{OH})_2$ and H_2SO_4 . This was merely a refinement of the rather crude removal of the ions in the previous step. The resultant solution, free of these ions, was found in every case (in every isolation procedure) to be of a definitely alkaline reaction when tested with litmus. This indicated that the glyoxalin or glyoxalines present were predominantly of a basic character, resembling, in this respect, Histidine. Estimation of the glyoxalin content gave a result of 408 mg. The loss in the phosphotungstate precipitation was therefore quite large - 237 mg.

The solution was now evaporated in vacuo to a small volume. When a volume of about 1 ml. had been

reached, a mass of crystals appeared in the tube. These were crystals of the glyoxalin as the free base. For the sake of greater purity of the final product, it was decided to isolate the material as a mono-hydrochloride salt. Thus, on the assumption that the material was histidine, the requisite amount of 1.0 N. HCl was added to the aqueous solution. Actually the amount of HCl used was slightly in excess of the theoretical value.

Decolorization - Since the solution contained some color (a light brown), it was decided to decolorize it by the use of Animal Charcoal. We were aware, at the time, of the work done by Niendorff⁽²⁵⁾ and the claim made, that histidine was completely removed from solution by shaking with Blood Charcoal. However, with the technique used, we found that very little adsorption of the glyoxalin occurred with the use of the activated blood charcoal. Repeated decolorization of a final concentrated solution of the glyoxalin (immediately prior to final isolation) involving the use of 1.5-2.5 grams of activated blood charcoal caused losses in the order of 2 or 3 milligrams out of a total of 400 or 500 milligrams. Therefore, losses due to decolorization of the final concentrated solution of glyoxalin with blood charcoal were quite negligible.

The procedure used in decolorizing the concentrated glyoxalin solution was as follows: The solution, in a test-tube, was brought to boiling and then about 0.5 gm. of blood charcoal was added. This mixture was boiled for a few seconds and then filtered through a small gravity

filter funnel. The greater part of the charcoal was retained in the tube for better washing. 2 or 3 c.c. of distilled water were added to the charcoal in the tube and the mixture boiled briskly for a few seconds and then decanted through the filter. The charcoal was washed 2 or 3 times in this manner. The resultant filtrate was quite clear and almost perfectly colorless.

Final Isolation - The decolorized solution was evaporated in vacuo to a small volume (about 1.5 ml.). Absolute Ethyl alcohol was added in small quantities with shaking till marked crystallization occurred. In some cases warming the solution to near-boiling, before addition of the ethanol, was found to facilitate crystallization. On the appearance of crystals the tube was stoppered and placed in the refrigerator for the completion of crystallization. After standing in the refrigerator for 1 or 2 days, the crystals were filtered off by suction through a small, sintered-glass funnel, washed first with 2-3 c.c. of 95% Ethanol and finally with 3-4 c.c. of absolute ethanol. The crystals were allowed to suck dry on the pump, then placed in the oven at 110°C . for $\frac{1}{2}$ -1 hour for completion of drying. The contents of the funnel were then transferred to a tared watch glass and weighed.

In this particular case, crystallization was very satisfactory, much more so than in many of the other isolations that were carried out. The yield of the monohydrochloride salt of the glyoxalin in the first

"crop" was 502.7 mg. out of a calculated possible yield of 552 mg. A Knoop test on this crude product gave a strong positive reaction. The melting point was 248-249°C. and the molecular color value was found to be 109.4 million mm. Therefore this material was, from all indications, relatively pure histidine.

Second and third "crops" were similarly obtained from the filtrate from the first "crop". The yields of these, in this case, were 22 and 36 milligrams respectively. They were, however, found to be quite impure and highly contaminated with creatinine. Diazo tests on these latter crops gave yellow colors of a weak intensity indicating low iminazole content. As a consequence they were discarded.

The above was the general procedure employed for the isolation of glyoxalin compounds from urine. The products obtained were crude and impure and for final identification further purification was necessary. As each "crop" was obtained several tests were carried out on it to determine both its nature and the degree of purity of the product. The tests used were:

- (a) The diazo test - A diazo reagent was made up and into it was dropped a crystal or two of the material obtained. The development of red color indicated relatively pure glyoxalin, whereas a yellow color indicated a high degree of contamination in which case the crop was usually discarded.

- (b) The Knoop test - This test was used to test for histidine. The Kapeller-Adler modification was used with but one exception - instead of removal of the excess bromine by Arsenious Acid, the Chloroform method of Hunter⁽²⁶⁾ was found preferable.
- (c) Melting point - The melting point of each crop was taken and compared to that of Histidine monohydrochloride (255-256°C.).
- (d) Molecular color value estimation - 5 mg. of each of the crops in question and of pure Histidine monohydrochloride were weighed out, dissolved and made up to 50 ml. and the molecular color value determined as outlined by Hunter⁽²¹⁾. The molecular color value of Histidine monohydrochloride is 113.1 million mm.

The latter two tests, (c) and (d), served as our criteria of purity of the product and on the basis of the results of these tests the decision was made whether or not to further purify the material. It was found necessary to recrystallize each crop at least once. This was carried out in the usual manner. The crop was dissolved in a minimal volume of distilled water, heated to boiling, decolorized with charcoal and filtered. The solution was evaporated, in vacuo, to a low volume and one or two drops of 1.0 N. HCl added. It was then

heated to near-boiling and then Absolute Ethanol was added till crystallization occurred. Subsequent steps were then the same as used for the isolation of a first crop. Recovery in the recrystallization was, as a rule, very satisfactory. In the example cited above, the first crop of 502 milligrams was recrystallized from water. The yield was 460 milligrams of a very pure product whose melting point was 255-256°C., the molecular color value was 109.4 million mm. and the Knoop test was strongly positive.

Further Notes on Procedure

It may at this point be advisable to discuss in greater detail some of the stages of the isolation procedure, especially in regard to some of the difficulties encountered.

The Silver fractionation procedure used was essentially the same as that described by Vickery and Leavenworth⁽²⁷⁾ except in the matter of a few obvious details. The authors employed Silver Oxide and Sulphuric Acid instead of Silver Nitrate, and they removed their 'Histidine fraction' within a pH range of 5.0-7.0. This range was later extended by the authors to pH 5.0-7.4. The results of these authors, with respect to the Silver fractionation curve of Histidine, were confirmed in the present study.

The Silver fractionation was applied to test portions of pure histidine solution and to a mercury fraction (T.M.R.(2)). Both solutions were made up to contain 1.5 mg. glyoxalin per ml. 7-5 ml. portions of each solution were taken and excess Silver Nitrate added in each case. The tubes were acidified by addition of two drops 5N Nitric Acid to each, and then Sodium Hydroxide (0.5 N.) was added to bring the contents of the tube to pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. The precipitates formed were removed, freed with hydrogen sulphide, the filtrates from the Silver Sulphide brought to definite volume and the glyoxalin content measured in each. The results for Histidine are shown in the table below:

(a) Pure Histidine Solution

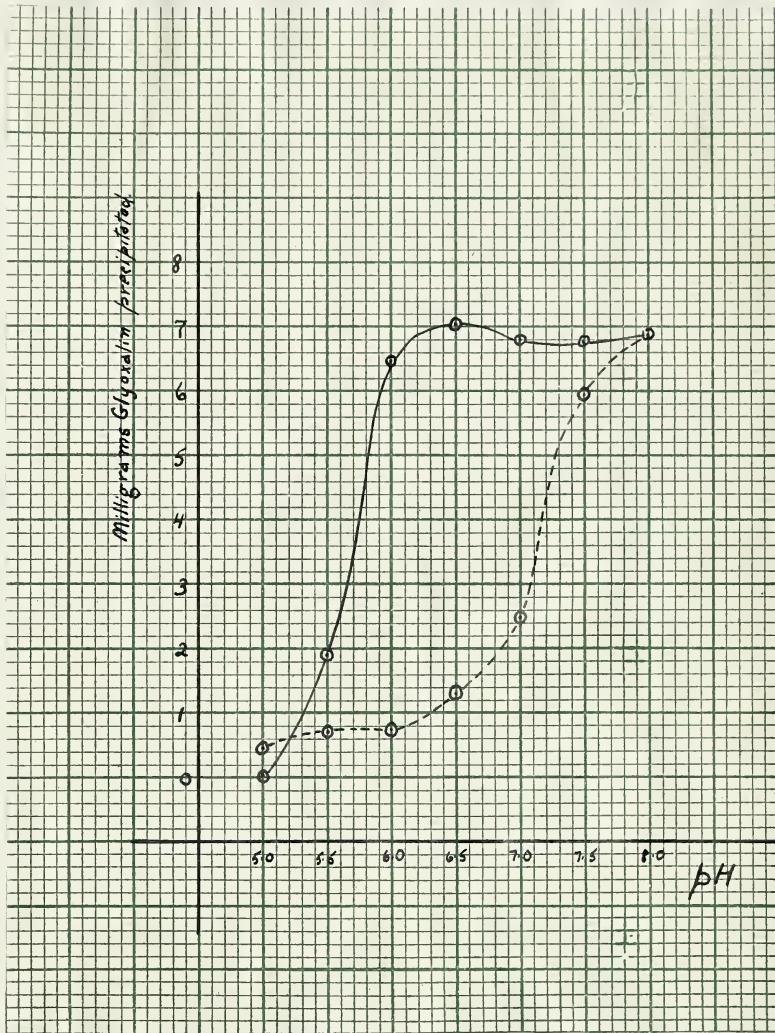
(Total content in each case = 7.5 mg.)

Tube	1	2	3	4	5	6	7
pH of sol'n	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Milligrams Histidine precipitated	0.04	1.95	6.50	7.02	6.80	6.80	6.90

(b) In a similar manner 7 tubes containing 5 ml. of the mercury fraction from urine (1.6 mg./ml.) were carried through the procedure.

Tube	1	2	3	4	5	6	7
pH of sol'n	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Milligrams Histidine precipitated	0.47	0.73	0.78	1.35	2.47	5.98	6.90

Graph I



Legend - Curve (a) _____

Curve (b) - - - - -

Curve (a)

Curve (b)

The results of (a) and (b) plotted on a graph (Graph I) showed a marked difference in the Silver precipitation curves of the two substances.

(c) Similarly a third series of this nature was carried out. Pure histidine was added to the Mercury fraction above to the extent of 1.5 mg./ml. In a similar manner 5 ml. portions were taken and treated in the same manner as the above. (Glyoxalin content on estimation - 17.68 mg./5 ml.). The results are tabulated below:

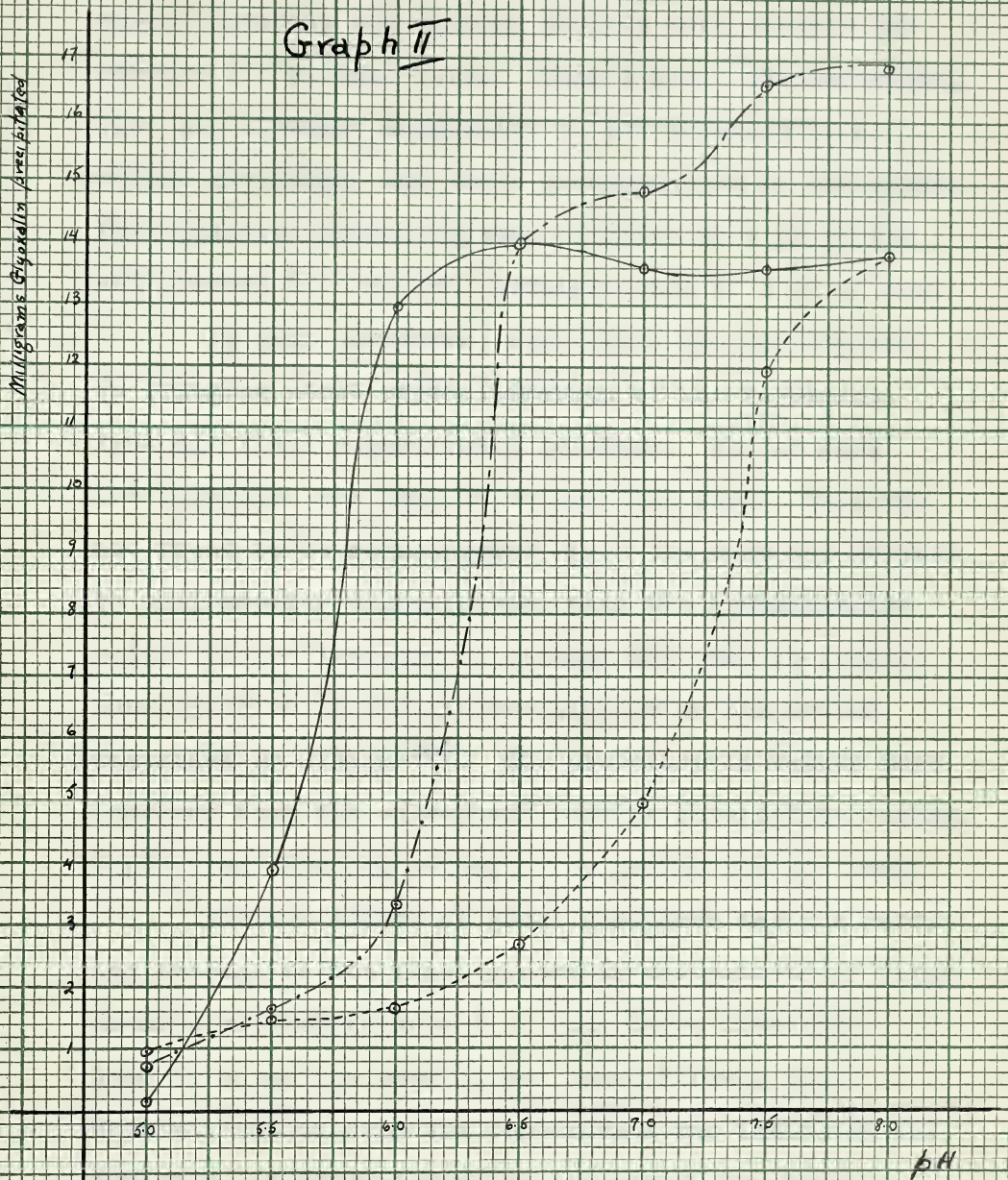
Tube	1	2	3	4	5	6	7
pH of sol'n	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Glyoxalin precipitated milligrams	0.75	1.69	3.38	14.00	14.82	16.64	16.90

These results have been shown on Graph II and compared with the results of (a) and (b), the values of the latter being doubled in this case, to allow for easier comparison.

Study of the (c) Curve brought some interesting observations. The curve lies between that of (a) and that of (b). It ascends steeply to a pH value of 6.5, indicating that the bulk of precipitation occurred between pH values of 5.0-6.5. This point coincides with the point of maximal precipitation of Histidine, this fact suggests that the glyoxalin being precipitated up to this point is histidine. The curve there dips

Graph II

Milligrams Glycadin Area Related



Curve (a) ————— Histidine
 Curve (b) - - - - - Urine
 Curve (c) — · — · — Urine + Histidine

rather markedly and finally ascends more steeply and reaches its maximal level. This dip and the succeeding rise are strongly suggestive of the presence of a second glyoxalin, one whose precipitation by the Silver fractionation method appears to start at a pH of 6.5 and proceed to a maximal precipitation of a pH of 8.0.

In some instances, difficulty was encountered in bringing about crystallization of the glyoxalin from the final concentrated solution. In one case the glyoxalin was removed as the free base. This was not found to be advantageous since the base was subject to much greater contamination than the hydrochloride salt. In one instance an attempt was made to isolate the glyoxalin as the dihydrochloride by the method described by Gilson⁽²⁸⁾. The procedure is rather more involved than the subsequent results obtained warranted. Aside from this one attempt, the method was not used.

Another source of great annoyance was the carrying through to the final glyoxalin concentrate of large amounts of Creatinine and in some cases Creatine. When this was the case it was found, firstly that crystallization from the concentrate was inhibited and secondly, if crystallization did occur, the crops invariably contained large amounts of the impurities. In one case a first crop obtained was found to consist purely of Creatine.

It was found to be advisable to remove these impurities before the isolation of the glyoxalin was attempted. Creatine was not dealt with specifically since Creatine is not precipitated by Phosphotungstic Acid. Creatinine, however, is precipitated by this reagent and so we expected it to be present in the final glyoxalin concentrate. Due, however, to the convertibility of Creatinine to Creatine or vice versa, a small amount of the latter could be expected to be present.

Creatinine was removed from the concentrate by precipitation as the monopicrate. The creatinine content of the concentrate was first colorimetrically determined by the method used for the estimation of that substance in blood. The requisite amount plus a 10% excess of purified picric acid was weighed out, dissolved in a small volume (1-2 c.c.) of water and added to the glyoxalin solution. The mixture was evaporated in vacuo to volume of 5-10 ml. and allowed to stand in the refrigerator overnight. The creatinine monopicrate was filtered off by suction, washed with a total of about 5 ml. of ice-cold distilled water and discarded. This creatinine monopicrate precipitate, on investigation, was found to contain insignificant amounts of glyoxalin, so that the danger of precipitation of the glyoxalin by the picric acid was not a factor.

The filtrate from the Creatinine picrate was freed of excess picric acid by addition of hydrochloric acid and extraction of the picric acid from the solution by Benzene. The amount of hydrochloric acid added varied somewhat - from 2 to 5 times the calculated amount necessary to convert the glyoxalin to the monohydrochloride. This was done in order to expedite the extraction of the picric acid by Benzene.

By the above method 92-95% of the Creatinine in the glyoxalin solution was safely removed, the loss of glyoxalin throughout the procedure amounting to approximately 5%. The success of the method depended upon maintenance of a low volume and maintenance of low temperature to limit the solubility of Creatinine monopicrate.

In one case, after the removal of Creatinine, the glyoxalin was precipitated as the flavianate, was dissolved in hot water, Barium hydroxide added and the Barium flavionate filtered off. Sulphuric acid was added to the filtrate to completely remove Barium. The solution after filtration was then made acid with Hydrochloric acid and the remainder of the flavianic acid removed by extraction with Butyl alcohol. The glyoxalin was then isolated in the usual manner, as the monohydrochloride. The product was found to be quite pure. The melting point was 254-255°C. A mixture of this substance and of pure histidine monohydrochloride melted at 256°C. The molecular color value was found to be 114 million mm.

Therefore, though the flavianic precipitation is involved, it is a useful procedure in purification of the glyoxalin.

Results of Isolation Procedures

In all, five urine samples were put through the procedure for the isolation of glyoxalines. The crystalline products when first obtained were in a variable state of purity, the degree being estimated by the performance of several tests in which the results constituted our criteria of purity. These tests have been mentioned previously (page 36). Those of immediate interest were (a) Melting points - both on the material alone and then on mixture with pure Histidine monohydrochloride, (b) Molecular color value and (c) the Knoop test.

The results of the various isolation^s performed are tabulated below under the heading of the sample of urine used:

(1) J. G. R.

Glyoxalin content of original urine = 815 mg.
 " " " final concentrate = 314 mg.
 Reaction - alkaline

Highly contaminated with creatinine. Several crops were taken off but all except one (46 mg.) were found to be largely creatinine. This crop, on recrystallization showed these properties:

- (a) Strongly positive, Knoop reaction
- (b) " " Diazo test

(c) Molecular color value = 109.5 million mm.

(d) Melting point - Simple = 254-256°C.

- Mixed - 255-256°C.

(2) T. M. R. - Sample (a)

Glyoxalin content of original urine = 546 mg.

" " " final concentrate = 203 mg.

Reaction - alkaline

Crystallization as the monohydrochloride yielded
125 mg. whose properties were:

(a) Strongly positive, Knoop test

(b) " " Diazo test

Molecular color value = 97.0 million mm.

(c) Melting point = 240°C.

(3) Sample (b)

Glyoxalin content in original urine = 630 mg.

" " " final concentrate = 495 mg.

Reaction - alkaline

Several crops were removed but were found to be
highly contaminated with Creatinine. The latter was
removed by the Picric Acid method. Content of glyoxalin
in final, creatinine-free filtrate = 120 mg.

Isolated - 99.3 mg. as Monohydrochloride.

(a) Strongly positive, Knoop test

(b) " " Diazo test

Molecular color value = 113 million mm.

(c) Melting points - Simple - 253-255°C.

- Mixed - 255-256°C.

(4) G. A. L.

Glyoxalin content of original urine = 1066 mg.

" " " final filtrate = 408 mg.

Reaction - Alkaline

Isolated as first crop 503 mg. as monohydrochloride.
 on recrystallization these properties showed:

- (a) Strongly positive, Knoop test
- (b) Molecular color value = 110 million mm.
- (c) Melting points - simple = 255-256°C.
 - mixed = 255-256°C.

The product was subjected to further tests:

	<u>In Free Base</u>	<u>In Monohydrochloride</u>
Nitrogen content -	27.46%	20.23%
	(Theoretical)	(Theoretical)
	(= 27.10%)	(= 20.05%)
Chloride content -	--	16.44%
		(calc. = 16.92%)

(5) Mrs. F.R. (Pregnancy Urine)

Glyoxalin content of original urine = 1615 mg.
 " " " final concentrate = 1088 mg.

Reaction - slightly alkaline

Isolated in four successive crops - 589, 86, 380
 and 135 mg. glyoxalin as the monohydrochloride salt.

Crop I - Recrystallized - split into 2 portions.

(a) Part I of 1st Crop

Yield = 348 mg.

Knoop test - strongly positive

Molecular color value = 113 million mm.

Melting points - simple = 253°C.
 - mixed = 253-255°C.

Analysis

Chloride = 16.48% (Calc. = 16.92%)

Nitrogen = 19.99% (Calc. = 20.05%)

(b) Part II of 1st Crop - This portion went through into the filtrate when the first part was crystallized out. Appeared as a second crop in the filtrate from the recrystallization of the main Crop I.

Yield = 178.3 mg.

Knoop test - negative

Molecular color value = 115 million mm.

Melting points = 265-267°C.

Crops II and III - mixed and recrystallized

Yield - 187.3 mg.

Knoop test - negative

Molecular color value = 114 million mm.

Melting point = 274-276°C.

Crop IV - crude - not recrystallized

Yield = 134.6 mg.

Melting Points = 230-234°C.

Molecular color value = 26 million mm. Impure.

The occurrence of these two instances of negative Knoop tests in products whose other properties so closely resembled Histidine was, to us, highly significant and was considered worthy of special attention. The further study of these Knoop-negative glyoxalin samples is discussed later under the heading of New Glyoxalin.

Identification of the Crystalline Products

Several tests were applied and several criteria satisfied before the substances isolated were identified. These tests, their application and significance may now be discussed in a more expanded form.

(a) Diazo Test - A strong positive diazo reaction was the first requisite. If the test, on application was negative, the material was discarded. The degree, character of development and quality of the final color were identical with the results obtained with Histidine.

(b) Molecular Color Value - This is a direct out-growth of the Diazo test. Various glyoxalins yield, in similar concentration, differing color intensities with respect to the color standard used.

Histidine	- 114.7	million	mm.	(Hunter ⁽²¹⁾ , 1925)
Histidine monohydrochloride-	113.1	"	"	"
Histamine	- 138.0	"	"	(Koessler and Hanke ⁽¹⁴⁾ , 1919)
Iminazole lactic acid	- 145.1	"	"	"
Iminazole propionic acid	- 191.0	"	"	"

The degree of color development is a characteristic of the individual glyoxalin and is, therefore, useful in estimation of the purity of the glyoxalin and as an aid in identification of the product.

(c) Melting Points - Reported melting points of histidine and of some of its salts are listed below:

d-Histidine	- 287-288° C. (33)
l-Histidine	- 265° C. (33)
dl-Histidine	- 285-286° C. (33)
l-Histidine Monohydrochloride	- 255-256° C. (30)
l-Histidine Dihydrochloride	- 245-246° C. (28)

The melting points of the products of the isolation procedures compared very favorably with the reported melting of Histidine Monohydrochloride and with the melting point of a known sample of the pure salt.

(d) Knoop Test - This has been previously explained and its specificity for Histidine in either the l- or d- form has been noted.

(e) Specific Rotation Studies - As a final check, to determine whether the histidine being excreted was of the d- or l- variety, specific rotation determinations were done. The procedure used was that described by Lutz and Jirgensons⁽³¹⁾.

l-Histidine has a specific rotation of -39.3° whereas l-Histidine monohydrochloride in an aqueous solution of equal concentration (M/20) has a specific rotation of $+2.3^{\circ}$. The low reading that would be obtained on the polarimeter with the material isolated led us to prepare the free base.

The free base was prepared as described by Abderhalden and Weil⁽³²⁾. The histidine (or glyoxalin) hydrochloride was dissolved in a small volume of distilled water and to it was added the calculated amount of Normal Lithium hydroxide solution. This mixture was evaporated to dryness in the vacuum desiccator and finally heated in the electric oven (110°C . for $\frac{1}{2}$ -1 hour). The Lithium chloride (and the

slight excess of Lithium hydroxide) was extracted with absolute ethyl alcohol. The residue, free of chlorides, was recrystallized from a water-alcohol mixture as used in the crystallization of the monohydrochloride. Care was taken to remove all chloride completely, by a second recrystallization if necessary, since the presence of small amounts of chloride could cause rather large error in the specific rotation figure.

M/20 solutions of the free base were used in each case. The polarimeter tube used was 1 decimeter in length. Readings were taken at room temperature (25°C.) and the source of light was the Sodium Vapor Lamp.

The procedure was carried out on three samples:

- (1) Histidine Monohydrochloride (pure salt - known)
- (2) Glyoxalin Hydrochloride (G. A. L.)
- (3) Glyoxalin Hydrochloride (F. R.) - Crop I, Part (1).

(1) Histidine Monohydrochloride - The pure base was prepared as described above. Melting point (uncorrected) = 276 C. Readings taken of an aqueous M/20 solution of the free base gave these results:

$$\alpha = -0.28^{\circ}$$

$$\left[\alpha \right]_0^{25^{\circ}} = \frac{-0.28^{\circ} \times 100}{0.77649 \times 1} = \underline{\underline{-36.06^{\circ}}}$$

$$\text{Reported } \left[\alpha \right]_0^{25^{\circ}} = -39.3^{\circ}$$

(2) Glyoxalin Hydrochloride (G. A. L.) -

Precisely the same procedure followed. Melting point of free base = 277-278°C.

$$\alpha = -0.309^{\circ}$$

$$\left[\alpha \right]_0^{25^{\circ}} = \frac{-0.309^{\circ} \times 100}{0.77542 \times 1} = -39.85^{\circ}$$

(3) Glyoxalin Hydrochloride (F. R.) - Crop I, Part (1)) - Melting point of free base = 276-277°C.

$$\alpha = -0.267^{\circ}$$

$$\left[\alpha \right]_0^{25^{\circ}} = \frac{-0.267^{\circ} \times 100}{0.77560 \times 1} = -34.31^{\circ}$$

The above results proved to our satisfaction that the substances that had been isolated, were beyond a doubt pure l-histidine.

"New" Glyoxalin

The specific rotation of the two samples that had given a negative anoop test were also determined. Both these samples were from the same urine (F. R.). These were, respectively, the second portion of the recrystallization product of the original first crop, henceforth referred to as A, and the recrystallized product of the combined second and third crops, referred to as B.

Lithium hydroxide (1 N.) was added to aqueous solution of both A and B, as in previous cases, for the removal of Chlorides. It was found, however, that on attempting to bring the solution to dryness,

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1. The Earth's crust is composed of various rocks and minerals.

$$P = \rho \cdot g \cdot h$$

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17. The Earth's crust is composed of various rocks and minerals.

crystallization did not occur but, in each case, a very viscous material resulted, comparable to a very thick syrup. This syrup was found to be completely soluble on addition of absolute ethyl alcohol so that obviously the extraction of the lithium chloride could not be carried out in the usual manner.

To obviate this difficulty, it was finally decided to carry out, on each sample, a phosphotungstic acid precipitation to separate the glyoxalin from the added Lithium. The free base was then recovered from the phosphotungstate precipitate in the usual manner. Considerable difficulty was encountered in bringing about crystallization of the glyoxalin (as the free base) and it was finally found necessary to evaporate the glyoxalin solution to dryness and heat to constant weight (at $110^{\circ}\text{C}.$) in weighed tubes. The yield of pure glyoxalin base after this procedure was:

A = 118.6 mg.

B = 141.4 mg.

Each was dissolved in boiled, distilled water and made up to 10 ml. These solutions were then used for the polarimeter. The results of the specific rotation readings taken of these two samples are given below:

A - That part of the first crop which on recrystallization passed on to the filtrate and was recovered as a second crop.

$$\alpha = +0.135^{\circ}$$

$$\left[\alpha \right]_0^{25^{\circ}} = \frac{100 \times 0.135}{1.186 \times 1} = +7.26^{\circ}$$

B - Recrystallized second and third crops.

$$\alpha = +0.030^{\circ}$$

$$\left[\alpha \right]_0^{25^{\circ}} = \frac{100 \times 0.030}{1.414 \times 1} = +2.12^{\circ}$$

These results might have been explained by the possible presence of a considerable admixture of d-histidine with the normal l-histidine. However, the Knoop test was negative and since both d- and l-histidine give a positive Knoop test, this possibility was eliminated from consideration. The behavior of the substances during the lithium hydroxide treatment was definitely atypical. Also, as stated above, the melting points of the hydrochloride salts were decidedly above the expected value. Further study of the material was fully indicated.

These two crops were mixed, the calculated amount of 1 N. Hydrochloric acid added and the glyoxalin isolated as the monohydrochloride salt. After twice recrystallizing from water, the final yield was 160 mg. This purified product was found to give no trace of color with the Knoop test. The melting point was found to be 267°C . The substances began to brown at 220°C ., became quite dark at 240°C . and contracted to a small black mass before melting when great expansion and

evolution of gas occurred.

An elementary analysis of this substance was done by Dr. Stanley Cook of Ayerst, McKenna and Harrison (Montreal). The following results were found:

Carbon	-	35.53%
Hydrogen	-	4.50%
Oxygen	-	22.72%
Nitrogen	-	20.46%
Chloride	-	16.79%

A fusion test with Sodium metal proved the absence of Sulphur and Phosphorus.

From the above results a tentative formula ($C_6H_{(8 \text{ or } 9)}O_3N_3 \cdot HCl$) was drawn up for the monohydrochloride salt of the glyoxalin. It appeared that this might be a hydroxy derivative of Histidine. Examination proved that the substance contained no free Amino-N, nor did it respond to the Ninhydrin test. Thus, definitely, the substance was not an amino-acid.

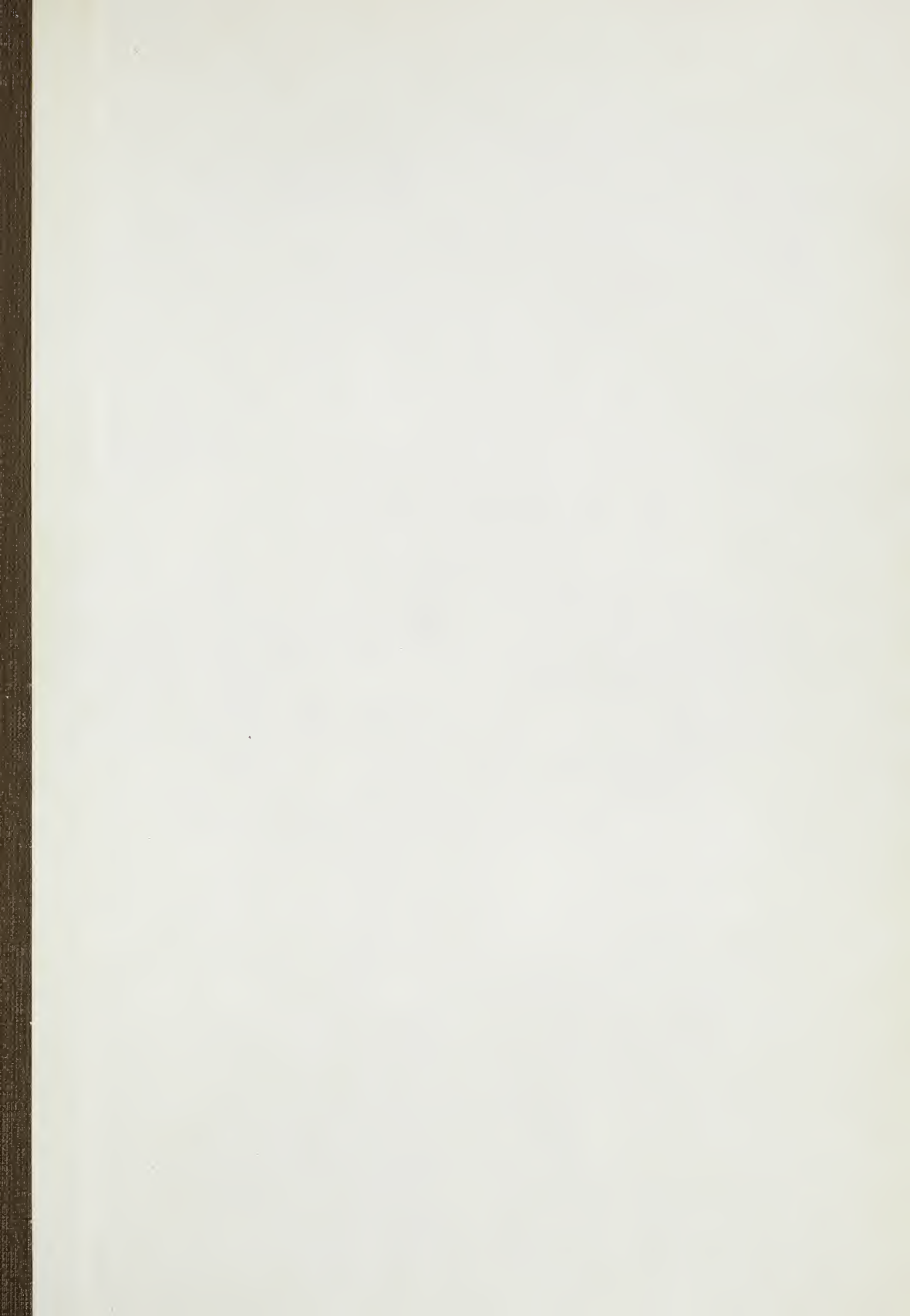
This substance, which has not yet been identified and has probably not been previously described, appears to be a large constituent of the glyoxalin content of normal human urine. The presence of this new substance in urine accounts for a number of discrepancies in the literature between the diazo and Knoop tests in urine, for while this substance gives a strong diazo reaction, the Knoop reaction is quite negative.

IV Conclusions and Summary

1. A new method has been described for the quantitative estimation of iminazoles in human urine.
2. The nature of these iminazoles has been studied.
1-Histidine has been isolated from several normal male urines and from one pregnancy urine.
3. A new glyoxaline is described whose properties are very similar to those of Histidine except that it gives a negative Knoop test. Its presence in urine probably explains much of the disagreement between various authors with respect to the diazo and Knoop tests in their application to urine.

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